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CARTILAGE INTERMEDIATE LAYER PROTEIN AND NUCLEIC ACIDS THEREFOR

CROSS-REFERENCE TO RELATED APPLICATION

This application relies on, and claims the benefit of the filing date of, U.S. Provisional Application Serial No. 60/142,054, filed July 2, 1999, the entire disclosure of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of molecular biology and protein biochemistry. More particularly, it relates to purification and characterization of proteins present in the matrix of cartilage-containing tissues, and to the cloning and characterization of genes encoding these proteins.

Description of the Related Art

Articular cartilage is a heterogeneous tissue in which cells are arranged in layers, forming a matrix. The layers have a different composition and function depending on their location with respect to the articular surface of the subchondral bone. The extracellular matrix is also arranged into compartments around the cells: pericellular (closest to the cell), territorial (extending around individual or groups of chondrocytes), and interterritorial matrix (farthest away from the cells). The matrix is produced by the chondrocytes and contains, as major constituents, fibril-forming collagens and large aggregating proteoglycans that are assembled into highly organized structures

(Heinegård, D. and Oldberg, Å., 1993, *In Connective Tissue and Its Hereditary Disorders* (Royce, P.M. and Steinmann, B., eds.) pp. 103-147, Wiley-Liss Inc., New York). Collagen confers tensile properties to the tissue, whereas proteoglycans play a key role in the normal resilience and load dissipation of the cartilage.

5 There is also a minor population of non-collagenous proteins for which no functional role has yet been identified. They might have roles in maintaining the tissue homeostasis by the regulation of matrix assembly, cell recognition, and cell attachment. They might also have a part to play in balancing the processes of cartilage repair and degradation, as well as in disease processes where degradation outbalances repair, and loss of tissue ensues. This third set of

10 matrix proteins are the non-collagenous glycoproteins, including several members of the family of leucine-rich repeat (LRR) proteins and the thrombospondins. The LRR proteins include decorin, biglycan, fibromodulin, and lumican, all with the capacity to bind to collagen (for refs. see Heinegård et al., 1998, "Biochemistry and metabolism of normal and OA cartilage", *In* Osteoarthritis, K.D. Brant, M. Doherty, and S. Lohmander, eds., Oxford University Press, New

15 York, In press). These molecules are found along surfaces of collagen fibrils in the tissue.

 Other members of this family include chondroadherin (Neame et al., 1994, "The structure of a 38-kDa leucine-rich protein (Chondroaherin) isolated from bovine cartilage", *J. Biol. Chem.* **269**:21547-21554) and PRELP (Bengtsson et al., 1995, "The primary structure of a basic leucine-rich repeat protein, PRELP, found in connective tissues", *J. Biol. Chem.* **270**:25639-25644). The

20 thrombospondin family includes the pentameric COMP, which is one of the more abundant cartilage matrix proteins (Hedbom et al., 1992, "Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage", *J. Biol. Chem.* **267**:6132-6136; Oldberg et al., 1992,

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progressing disease, it is desirable to detect the disease early in its development and treat it before extensive damage has occurred. There exists a need for methods and compositions for early detection of this clinically important disease. Possible markers for osteoarthritis, which could be used early in the development of the disease, include the matrix proteins.

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SUMMARY OF THE INVENTION

One such matrix protein is the protein of the present invention. This protein, referred to herein as Cartilage Intermediate Layer Protein (CILP), has been identified and purified from human articular cartilage. The present invention describes the isolation and characterization of a CILP that is a 91.5 kDa, single-chain protein from human articular cartilage. The invention also describes methods of making CILP, portions (fragments) of CILP, and analogs and homologs of CILP.

Further, the present invention provides methods of using the proteins (and fragments) of the invention, including use in early detection of OA, treatment of individuals suffering from joint disease, such as OA, use in identifying other cartilage matrix proteins, and use in identifying the biochemical and biophysical bases of OA. An additional use of the protein or protein fragments of the invention is in the production of antibodies specific for the protein.

Thus, the present invention includes antibodies that specifically react with CILP, immunogenic fragments of CILP, and cross-reactive analogs and homologs of CILP. Using the antibodies of the present invention, other CILP-related proteins can be identified and isolated.

The present invention also provides nucleic acids encoding the protein of the invention, portions (fragments) of the protein, or analogs or homologs of the protein. In addition, the

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invention provides other nucleic acids encoding all or part of CILP, such as messenger RNA (mRNA) encoding all or part of CILP, as well as probes and primers for identifying, isolating, and characterizing other CILP proteins, analogs, and/or homologs. Having provided nucleic acids encoding the protein of the invention, or parts thereof, the invention also provides methods of isolating, or otherwise producing, the nucleic acids of the invention.

Methods of using the nucleic acids are also provided by this invention. Included in these methods are methods for producing the proteins of the invention, as well as methods of producing portions of the proteins, such as immunogenic portions. The nucleic acids of the invention can also be used in methods of treatment, such as therapeutic methods for treatment of OA. Methods of detecting, isolating, and purifying nucleic acids encoding analogous or homologous proteins are provided through the use of probes and primers according to the present invention. Methods of identifying genes involved in OA are enabled by the present invention, as are transgenic animals in which CILP expression is controlled or eliminated.

An additional aspect of the invention is a kit containing reagents necessary for making or using the protein of the invention, making or using the nucleic acid of the invention, or making and using the antibodies of the invention. For example, the nucleic acids of the invention can be supplied in a kit, for both *in vivo* and *in vitro* applications, or the antibodies of the invention can be supplied in a kit for detection of abnormal levels of CILP or immunoreactive fragments or analogs of CILP.

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Figure 1 shows the results of an immunoblot analysis of CILP in extracts of articular cartilage of different species.

B. Immunoblot of the gel from A, using antiserum against the human CILP.

Figure 2 shows the results of an ELISA of CILP in human tissues.

B. Cartilages: tracheal (■), articular (●), rib/costal (▲).

For both **A** and **B**, purified CILP is represented by open circles.

Figure 3 shows CILP content in articular cartilages from individuals of different ages. All samples were analyzed in triplicate, and the mean value was used for calculations.

Figure 4 shows the immunolocalization of CILP in human articular cartilage (hip).

Polyclonal antibodies to CILP were used.

A. Sections were pre-digested with testicular hyaluronidase before incubation with anti-CILP antibodies.

B. Sections were pre-incubated with pre-immune sera.

Figure 5 shows the CILP content in different layers of human articular cartilage.

Figure 6 shows synthesis of CILP by articular chondrocytes in explant cultures.

- SH indicates non-reducing SDS-PAGE; + SH indicates reducing SDS-PAGE.

Figure 7 shows the results of deglycosylation of CILP by *N*-glycosidase F digestion (8% SDS-PAGE/Coomassie).

Figure 8 shows CILP nucleotide (SEQ ID NO:1) and translated amino acid (SEQ ID NO:2) sequences. The putative signal peptide is boxed. The arrows indicate the putative cleavage site. The underlined amino acids represent peptides that were sequenced (see Table 1). The cysteines are circled, and the putative N-glycosylation sites are marked with a star. The stop codon is marked and the polyadenylation signals are underlined. The sequence homologous to porcine NTPPHase is shown in italics beginning at residue 683. The Type I thrombospondin repeat sequence consensus is identified at residues 131-157, with conserved residues underlined.

Figure 9 is a schematic representation of the organization of the CILP cDNA. The scale in kb is shown at the top. The second line displays the cDNA size. The locations of the restriction sites are indicated. ATG is the first initiation codon for translation, TAA is the termination codon for translation, [A]_n indicates the location of the poly(A) tail. The overlapping

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cDNA clones and the genomic clone are shown with respect to the schematic structure of the cDNA. The expression construct is shown at the bottom, with the different clones used.

Figure 10 shows the results of Northern blot analysis of CILP mRNA. Total RNA (10 µg) extracted from human articular chondrocytes were analyzed by Northern blotting using the [α³²P]dCTP probe of a 1585-bp *BsmI/EcoRI* fragment from the 92C-1 clone. RNA size standards are indicated on the left.

Figure 11 shows the expression of the full-length cDNA *in vitro* and in COS-7 (*in vivo*).

A. pBluescript II KS(+) with the expression construct was *in vitro* transcribed-translated by the TNT[®] T3 Coupled Reticulocyte Lysate Systems in the presence of [³⁵S]methionine. The *in vitro* synthesized polypeptide was resolved on a 4-16% gradient SDS-Polyacrylamide gel and visualized by fluorography. Lane 1: Control plasmid only. Lane 2: Plasmid with the expression construct.

B. COS-7 cells were transfected with the pSVL vector containing the expression construct and grown in a serum-free medium containing [³⁵S]methionine. After labeling, the cell extract was immunoprecipitated with the antibodies against CILP. The immunoprecipitated material was resolved on a 4-16% gradient SDS-Polyacrylamide gel and visualized by fluorography. Lane 1: Transfection with the vector only. Lane 2: Transfection with the vector containing the expression construct. The molecular weight standards are indicated on the left.

C. COS-7 cells were transfected with the pSVL vector containing the expression construct and grown in a serum-free medium containing [³⁵S]methionine. After labeling, the medium was immunoprecipitated with the antibodies against CILP. The immunoprecipitated material was resolved on a 4-16% gradient SDS-Polyacrylamide gel and visualized by

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joined together through amide bonds. Peptides are two or more amino acid monomers long. Polypeptides are more than ten amino acid residues in length. Proteins are more than thirty amino acid residues in length. Thus, peptides include polypeptides and proteins, and polypeptides include proteins. Standard abbreviations for amino acids are used herein.

5 Analog includes all proteins, polypeptides, or peptides that show substantial chemical and functional similarity to CILP, and can include CILP with post-translational modifications not necessarily present in the CILP as purified (*e.g.*, glycosylation at sites not glycosylated in the protein as purified, or proteins containing no glycosylation at sites ordinarily glycosylated), or as found in nature. In embodiments, the analogs comprise CILP that has been chemically modified. In preferred embodiments, the CILP has been modified to improve a desirable characteristic, for example, immunogenicity, cell adhesion, cell permeability, or stability. For example, cyclic peptides can be made to optimize the interaction site. In other embodiments, the analog is modified such that it can be detected more readily, for example, by including a label in the modified structure. Chemical modification can include any type of modification to the base molecule envisioned by the skilled artisan, as long as the resulting molecules retains essentially all of its assayable characteristics. In other words, the molecule must still be recognizable, by standard biochemical assays used in the art, as a CILP. Analog can have multiple additional functions beyond those of natural CILP, including non-natural enzymatic and/or cell binding activity, or can have fewer functions.

20 Homologs include CILP from all mammals, including, but not limited to, humans, bovine, murine, monkeys, apes, ungulates, felines, and canines. Homologs also include those of insects and lower eukaryotes (*e.g.*, yeast) as well. Homologs also include all proteins showing

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The proteins, polypeptides, or peptides of the invention, can be isolated and/or purified from any eukaryote in which they are naturally present, including, but not limited to, mammals such as humans, dogs, cats, monkeys, apes, horses, pigs, and rodents (*e.g.*, mice and rats). For example, because CILP was found to be restricted in its tissue distribution to cartilage and furthermore, to specific zones within the tissue (the precursor protein is synthesized by chondrocytes and processed close to secretion), it can be purified from chondrocyte-containing tissues. Purification techniques can include any of those known to the skilled artisan, in any combination or order, that results in purification of the desired polypeptide or peptide. A preferred technique is disclosed in the Examples; however, modifications of the method are contemplated by the invention as well.

Techniques for production of proteins, polypeptides, and peptides in high amounts by genetic engineering techniques through the use of expression vectors, such as plasmids, phages, and phagemids, are well known. The proteins and fragments of the present invention can be produced by insertion of the appropriate polynucleotide into an appropriate expression vector at the appropriate position within the vector. Such manipulation of polynucleotides is well known and widely practiced by the ordinary artisan. The proteins and fragments can be produced from these recombinant vectors either *in vitro* or *in vivo*, for example, in prokaryotic cells or lower eukaryotes (*e.g.*, yeast). In a preferred embodiment, the method for the production of a polypeptide of the invention comprises the steps of:

(a) optionally amplifying the polynucleotide coding for the desired protein or fragment using a pair of primers according to the invention by a target nucleic acid amplification method;

(b) inserting the nucleic acid of interest in an appropriate vector to create a recombinant vector;

(c) transforming or transfecting a host cell with the recombinant vector to create a recombinant (transformed) cell;

(d) culturing the recombinant cell in an appropriate culture medium;

(e) optionally separating the culture medium from the recombinant cell mass,

(f) optionally lysing the recombinant cells;

(g) optionally separating or purifying, from the culture medium, or from the pellet of the resultant recombinant cell lysate, the thus-produced protein or fragment of interest;

(h) optionally, characterizing the produced protein or fragment of interest.

Accordingly, the invention provides recombinant CILP proteins, polypeptides, and peptides.

In embodiments, the method of producing the protein or fragment can be used to facilitate a method of purifying the protein or fragment, or can be a method of purifying the protein or fragment as well. The method can further include quantifying the purified protein. In addition, many techniques for purifying recombinantly-produced proteins and fragments are well-known to the skilled artisan and, thus need not be detailed here. In general, these techniques result in separation of the CILP from at least one other macromolecule originally found in a sample containing the CILP, analog, homolog, or fragment. In preferred embodiments, the techniques result in separation of the CILP, analog, homolog, or fragment from all, or essentially all, other macromolecules and/or organic molecules originally found in the sample containing the CILP.

Macromolecules include, but are not limited to, polymers (*e.g.*, proteins, nucleic acids,

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polysaccharides), aggregates, membranes or membrane components (*e.g.*, lipid structures), and organelles. A CILP, analog, homolog, or fragment is essentially purified when no other macromolecules are present in the sample containing the CILP, analog, homolog, or fragment. A CILP, analog, homolog, or fragment is purified when it is the only component detectable in a sample, using standard detection methods. In preferred embodiments, the CILP, analog, homolog, or fragment is purified.

Techniques for isolation and purification include filtration, centrifugation, chromatography, and precipitation, and, in embodiments, are used to purify the protein or fragment of the invention. In embodiments, tags, such as affinity tags, are included in the protein to facilitate purification. Preferably, the tags are cleavable. Preferred techniques are described herein.

Chemical and enzymatic cleavage of proteins, and subsequent purification of the cleavage products, are well-known techniques that can routinely be applied to the proteins and polypeptides of the invention. Included in these techniques are those that cleave the polypeptides at an internal site (*e.g.*, an endopeptidase) and those that digest the polypeptide from one or the other end (*e.g.* an exopeptidase). Having the sequence of Figure 8 (and SEQ ID NO:1 and SEQ ID NO:2), the skilled artisan can use these techniques, or combinations of these techniques, to create fragments having the exact sequence desired. Furthermore, techniques are available for chemically modifying the isolated fragments to create, for example, highly immunogenic molecules or molecules with enhanced activities.

In embodiments, fragments are short stretches of contiguous amino acids according to Figure 8 (SEQ ID NO:2). These peptide fragments can be as short as 4 residues, for example

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Other applications might require an oligonucleotide that is, optimally, at least 30 nucleotides in length, such as an oligonucleotide that is approximately 50 nucleotides in length, approximately 100 nucleotides in length, or even more (*e.g.*, 150, 200, 250, 300).

5 The oligonucleotides can be used to identify the presence and concentration of CILP-encoding genes or mRNA, or both, both *in vivo* (*e.g.*, *in situ* hybridizations) and *in vitro* (*e.g.*, Southern and Northern blot analyses). They can also be used to identify analogs and homologs of the sequences presented in Figure 8 (SEQ ID NO:1 and SEQ ID NO:2) using known detection and screening methods, as well as study the regulation of expression of CILP and its related proteins during progression and/or treatment of joint disease.

10 In preferred embodiments of this aspect of the invention, recombinant vectors are provided, which contain the polynucleotides of the invention. The recombinant vectors can be any one known in the art, including, but not limited to, shuttle vectors, expression vectors, suicide vectors, and integration vectors. Exemplary vectors include plasmids, phages, phagemids, cosmids, and yeast artificial chromosomes (YACs). The recombinant vectors can be
15 used to express the polypeptides and peptides of the invention both *in vitro* and *in vivo*, as well as to transfer the nucleic acids of the invention into host genomes. In one particular embodiment of the invention, the vectors are used in therapeutic methods, such as a method of gene therapy. In other embodiments, the vectors are used to create transgenic animals, which can be useful for the study of joint disease and the role CILP and its analogs and homologs play in the disease. For
20 example, the transgenic animal can be used to develop and test potential drugs that ameliorate or otherwise treat OA, rheumatoid arthritis, crystal deposit arthritis, psoriatic arthritis, or reactive arthritis.

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5 In other embodiments of the invention, compositions comprising polynucleotides and/or recombinant vectors of the present invention are provided. Gene targeting techniques can be used to introduce therapeutic polynucleotides and vectors into host cells. One of the preferred targeting techniques according to the present invention consists of a process for specific replacement, in particular by targeting the CILP gene *in vivo*. Such a DNA targeting technique is well known in the art and often is referred to as a "knock out" technique. According to the present invention, "knock in" techniques are contemplated as well.

10 Accordingly, the invention provides recombinant cells comprising the polynucleotides, vectors of the invention. The invention also provides recombinant cells comprising the proteins or fragments of the invention. The recombinant cells are host cells into which the proteins, fragments, polynucleotides (including oligonucleotides), and vectors of the invention have been introduced. Recombinant cells include progeny (*i.e.*, offspring, daughter cells, cells directly descendent from the original transformant). The recombinant cells can be used for many purposes, including, but not limited to, expression of the proteins and fragments of the invention for purification and subsequent use in raising antibodies or treating individuals, producing a transgenic animal for the study of joint disease or for production of therapeutic compounds, and amplification of the nucleic acids, vectors, proteins, and protein fragments for use in therapeutic compositions.

20 Another aspect of the present invention is a kit. The kit can contain at least one protein or fragment of the invention, at least one polynucleotide or recombinant vector of the invention, and/or at least one antibody of the present invention. The kit can include one of the molecules of the invention, or multiple molecules, in any ratio or formulation. Preferably, the molecules of the

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present in the sample. Oligonucleotide primers can also be used to amplify and sequence the CILP mRNA to determine whether the CILP message encodes a mutant form of the protein.

EXAMPLES

The invention will be further clarified by the following Examples, which are intended to be purely exemplary of the invention, and should not be construed as limiting the invention in any way.

Example 1: Extraction of Cartilage.

Human articular cartilage was obtained at surgery after total hip replacement for femoral neck fracture. The cartilage was dissected clean, sliced into fine pieces and disrupted using a high speed homogenizer (Polytron, Kinematica GmbH, Kriens-Lusern, Switzerland) in 12 volumes (w/v) of 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4, also containing a protease inhibitor cocktail (5 mM benzamidine hydrochloride, 0.1 M 6-aminohexanoic acid), and 5 mM N-ethylmaleimide, as described by Heinegård and Sommarin, ("Isolation and characterization of proteoglycans", 1987, *Methods Enzymol.* **144**:319-372). The mixture was pre-extracted 4 hours at 4°C then centrifuged at 20,000 X g and 4°C for 30 minutes. The pellet was extracted with 12 volumes (w/v) of 4 M guanidine hydrochloride (GdnHCl), 0.05 M sodium acetate, pH 5.8, containing the protease inhibitor cocktail, 5 mM N-ethylmaleimide and 10 mM EDTA, for 24 hours at 4°C followed by centrifugation at 20,000 X g at 4°C for 30 minutes.

Proteins in the extract were separated from proteoglycans by CsCl-density gradient centrifugation with a starting density of 1.5 g/ml under dissociative conditions 4 M GdnHCl

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(Heinegård and Sommarin, 1987, *supra*). The gradient tube was divided into 4 equal fractions using a Beckman tube slicer, and the top two fractions (D3 and D4) were used for subsequent purification.

Example 2: Protein Purification.

The pooled fractions from the CsCl-gradient (D3 and D4) were concentrated by ultrafiltration (PM-10 membrane, Amicon, Inc. Beverly, MA, USA), followed by diaflow against 7 M Urea, 20 mM Tris-HCl, pH 8, and then chromatographed on a column of DEAE-cellulose (5 x 10 cm, DE52, Whatman, Maidstone Chemicals, Kent, United Kingdom) equilibrated with the urea buffer. After sample loading, the column was washed with 5 bed volumes of the equilibration buffer, and eluted with a linear gradient (2 x 800 ml) from 0 to 1 M NaCl. Selected fractions were analyzed by SDS-PAGE for the presence of CILP. One pool of the protein was bound to the DEAE column and eluted at 0.04 M of NaCl, while the rest of the protein was in the flow-through. This flow-through material was mixed with an equal volume of 20 mM sodium acetate, and the pH was adjusted to 5. Then the protein was further purified on a CM 52 column (1.6 cm x 15 cm, Carboxymethyl-cellulose, Whatman Maidstone Chemicals, Kent, United Kingdom), equilibrated in 7 M Urea, 10 mM sodium acetate pH 5 at 20 ml/hr. After loading the sample, the column was washed with 5 bed volumes of equilibration buffer followed by elution with a linear gradient of 0 to 0.5 M NaCl. Fractions of 10 ml were collected, monitored for protein content by measuring their absorbance at 280 nm, and analyzed by SDS-PAGE.

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5 The fractions containing CILP were pooled and concentrated by ultrafiltration, followed by diaflow against 4 M GdnHCl, 50 mM sodium acetate, pH 5.8 and further chromatographed on two serially coupled FPLC gel filtration columns of Superose 6 and Superdex 200 (Pharmacia Biotech, Uppsala, Sweden) equilibrated and eluted at 0.2 ml/min with 4 M GdnHCl, 50 mM sodium acetate, pH 5.8. Fractions of 0.5 ml were collected and monitored for protein content by measuring their absorbance at 280 nm. Protein patterns were analyzed by SDS-PAGE.

10 Early attempts to purify CILP from human articular cartilage involved direct extraction of the cartilage with the GdnHCl solution. However, the abundant albumin in the preparation masked a pool of CILP that was bound to the DEAE column. This pool eluted at a very low salt concentration (0.04 M NaCl), while the remainder of CILP appeared in the flow through, as discussed above. Preliminary studies were done using protein in the 0.04 M NaCl pool purified by CM52 chromatography and gel filtration. In a subsequent study investigating the component which bound to DEAE, the tissue was pre-extracted with phosphate buffered saline at pH 7.4, containing a protease inhibitor mix, to remove the albumin and other proteins not tightly held in the cartilage matrix.

15 The two pools of CILP, *i.e.* the one bound to the DEAE and the one in the flow through, showed identical peptide patterns after trypsin digestion when analyzed by reverse phase HPLC (C-18) (data not shown). The two protein variants also appeared to have a similar content of N-linked oligosaccharides since their respective mobilities upon SDS-PAGE before and after N-glycanase digestion were identical (data not shown). No further experiments were done to elucidate the difference between the two proteins. All the biochemical studies were done using

the protein pool from the CM-cellulose chromatography, even though the two pools appeared to contain the same protein.

Example 3: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Electrophoresis was performed as described by Laemmli (Laemmli, U.K., 1970, *Nature* 227:680-685) on gradient polyacrylamide (4-16%) slab gels with a 4% polyacrylamide stacking gel. Samples of the fractions were precipitated with 10 volumes of ethanol containing 50 mM sodium acetate and recovered after centrifugation as described elsewhere (Paulsson, M., Sommarin, Y. and Heinegård, D., 1983, *Biochem. J.* 212:659-667). Precipitates were dissolved in electrophoresis sample buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 0.002% Bromophenol blue and 20% Glycerol) either with or without 10% 2-mercaptoethanol, incubated at 37°C for 30 minutes, boiled at 100°C for 4 minutes, and electrophoresed. Gels were stained with Coomassie Brilliant Blue R (Serva, Heidelberg, Germany). CILP was pure as judged by this sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By this procedure a yield of 129 µg of CILP per gram of tissue wet weight was obtained. The relative mobility of CILP on SDS-PAGE was not significantly affected by reduction, which suggested a monomeric protein (data not shown).

The molecular mass of the purified CILP protein was estimated by running linear SDS-PAGE with concentrations ranging from 6% to 12% polyacrylamide (*i.e.*, 6, 8, 10, and 12% SDS-PAGE) (Heinegård, D. and Sommarin, Y., 1987, *supra*). A Ferguson plot was constructed (Ferguson, K.A., 1964, *Metabol. Clin. Exp.* 13:985-1002) from the relative mobilities of CILP and reference proteins. Molecular mass was determined from the retardation coefficients (K_1) as described by Banker and Cotman (Banker, G.A. and Cotman, C.W., 1972, *J. Biol. Chem.*

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Immunoblot

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Enzyme-linked immunosorbent assay (ELISA).

The assays were performed essentially as previously described (Larsson, T., Sommarin, Y., Paulsson, M., Antonsson, P., Hedbon, E., Wendel, M., and Heinegård, D., 1991, *J. Biol. Chem.* **266**:20428-20433). CILP was coated onto NUNC gamma-irradiated polystyrene microtiter plates (Immunoplate I, NUNC, Roskilde, Denmark) at 0.5 µg/ml in 4 M GdnHCl, 50 mM sodium carbonate, pH 10, by incubation overnight at room temperature (approximately 22°C). After rinsing with 0.15 M sodium chloride, 0.05% (w/v) Tween 20, the plates were coated with 2 mg/ml bovine serum albumin (Serva, Fine Biochemicals, Heidelberg, Germany) in phosphate buffered saline, pH 7.4, for 1 hour.

Samples of human tissues were extracted with 15 volumes of 4 M GdnHCl containing protease inhibitors as described above. The extracts were precipitated twice with ethanol (Paulsson, *et al.*, 1983, *supra*) and resuspended in 0.8% SDS in 10 mM phosphate buffered saline pH 7.4, to give stock solutions corresponding to 4 mg of the original tissue wet weight per ml. Dilutions were made from these stock solutions and added to an equal volume of antiserum in 4% (v/v) Triton X-100, 0.01 M sodium phosphate, pH 7.4. After pre-incubation for one hour at room temperature, this mixture was added to the coated wells of the microtiter plates. After 1 hour, plates were rinsed and bound antibodies were detected using a swine anti-rabbit alkaline phosphatase conjugate (Orion Diagnostica, Helsinki, Finland) with *p*-nitrophenyl phosphate (Sigma Chemical Co, St. Louis, MO, U.S.A.) as the substrate.

A standard curve using purified CILP in 0.8% (w/v) SDS, 10 mM phosphate buffered saline pH 7.4, was included in each microtiter plate. All samples were analyzed in triplicate and the mean value was used for calculations.

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The cartilage was frozen, embedded in O.C.T. compound (Tissue Teck II, Miles laboratories,

5 Naperville, IL, USA) and sectioned at -25°C in a cryostat. Cryosections (5 µm) were transferred
onto gelatin-coated glass slides, dried at room temperature for two hours, fixed at 4°C with cold
acetone for 10 minutes, and re-hydrated in phosphate buffered saline (PBS). To increase
antibody permeability, the sections were digested for 30 minutes at room temperature with 2
mg/ml of hyaluronidase (from bovine testes, type I, Sigma, St. Louis, MO, USA) in PBS, pH 5.
Endogenous peroxidase was quenched by incubating in PBS containing 1% (v/v) H₂O₂ for 20
minutes at room temperature. After rinsing three times with PBS-0.1% (w/v) BSA, each section
was incubated with goat-serum diluted 1:70 in PBS-0.1% (w/v) BSA for 20 minutes to reduce
non-specific binding. Sections were then incubated with the primary antibody against CILP
(diluted 1:800 with PBS-0.1% BSA) or with preimmune rabbit serum (diluted 1:400 with PBS-
0.01% BSA) at 4°C overnight in a moist chamber. After rinsing three times with PBS-0.01%
BSA, the sections were treated with biotinylated second antibody (diluted 1:200) and avidin
peroxidase conjugate using the vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA),
according to the protocol of the manufacturer.

The protein was detected primarily in the middle zone, while it was less prominent or
absent at the superficial and the very deepest zones of the articular cartilage (Figure 4). The
antibodies primarily stained the interterritorial matrix of the tissue, while low or no reactivity was
seen in the pericellular matrix of the chondrocytes. Based on its immunolocalization this protein
was named Cartilage Intermediate Layer Protein (CILP).

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To determine whether the protein is a true cartilage constituent synthesized by the chondrocytes, human knee articular cartilage explants were metabolically labeled with [³H]leucine and [³⁵S]sulfate. Human articular cartilage (femoral head) was obtained at surgery for hip replacement, dissected under sterile conditions and placed (25 mg tissue/ml medium) in Ham's F-12 culture medium (Gibco BRL, Grand Island, NY, USA), pH 7.4, supplemented with

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10% (v/v) fetal calf serum and 25 µg/ml of ascorbate. The explants were metabolically labeled with 50 µCi/ml of [³H]leucine and 25 µCi/ml of [³⁵S]sulfate (Amersham International, Bucks, UK) for 4 hours at 37°C under 5% CO₂ : 95% air. After labelling, the explants were washed with medium without isotopes, wiped dry, and extracted with 4M GdnHCl, as described above. An aliquot of the extract was precipitated with ethanol and dissolved in 60 µl of 10 mM phosphate buffered saline, pH 7.4, containing 0.8% (w/v) SDS. To bind excess SDS the sample was then mixed with 60 µl of 2% (v/v) Triton X-100 in the same buffer. Rabbit immune serum (30µl) was immediately added and the sample was incubated overnight at 4°C. Immunoglobulins and bound antigen were then adsorbed onto protein-A Sepharose (30µl) (Pharmacia, Uppsala, Sweden) by incubation for 4 hours at 4°C. Bound material was recovered by centrifugation, washed, resuspended in electrophoresis buffer, and electrophoresed as above.

Radiolabelled proteins were detected by fluorography. The polyacrylamide gels were washed in distilled water for 30 minutes, soaked in 5 volumes of 1.3 M sodium salicylate for 35 minutes, dried on a gel drier (LKB Bromma, Sweden), and exposed to a preflashed Kodak XAR-5 film for 3 - 4 weeks at -80°C.

As shown in Figure 6, a major band with a relative mobility corresponding to CILP was identified by fluorography after SDS-PAGE under reducing and non-reducing conditions. However, after reduction, two higher molecular weight components were also immunoprecipitated. They might represent either aggregates of CILP or possibly cross-reactivity with some related proteins or nonspecific reactivity that is sometimes observed at that position with other rabbit antisera (data not shown).

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Example 8: Cloning and Sequencing of the Gene Encoding CILP.

In general, all the molecular biological procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing, were performed by standard methods (Sambrook et al., 1989, Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

RNA extraction.

Human articular cartilage was obtained at surgery after total hip replacement, kept in PBS during dissection, shaved, and frozen in liquid nitrogen. Total RNA was extracted essentially as described by Adams *et al.* (Adams, M.E., D.Q. Huang, L.Y. Yao, and L.J. Sandell, 1992, "Extraction and isolation of mRNA from adult articular cartilage", *Anal. Biochem.* **202**:89-95) but omitting the CsTFA ultracentrifugation step. Instead, the RNA was precipitated twice with 1 vol of 100% isopropanol, then further purified with a RNA easy kit (QIAGEN Inc., Chatsworth, CA, USA) according to the manufacturer's protocol. The mRNA was purified from this preparation using the Oligotex mRNA kit (QIAGEN Inc., Chatsworth, CA, USA) by following the manufacturer's protocol.

cDNA probe generation and library screening.

Total RNA isolated from human articular cartilage was used as the template for the synthesis of the first-strand cDNA. Total RNA (20 µg) was reverse-transcribed using 400 Units of Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a 50 µl reaction mixture containing 100 ng of oligo(dT), 300 ng random hexamer primers, and 1 mM dNTP, for 1

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SK(+) containing the insert was rescued from the ZAP vector by the use of *in vivo* excision as described in the ZAP-cDNA[®] synthesis kit (Stratagene, La Jolla, CA, USA). The plasmid was digested with *XbaI/XhoI* and the cDNA size (2.3 kb) was determined by electrophoresis on a 1% agarose gel (NuSieve GTG, FMC Corp.).

5 Restriction fragments of the cDNA were generated with *Bam*HI and subcloned into a pCR-Script Amp SK(+). Sequencing was performed as described above, and compression artifacts were resolved using the dITP kit (USB, Cleveland, Ohio, USA). The sequencing primers used included T3 and T7 universal primers and internal sequencing primers. The 2.3 kb cDNA subclone was used to generate a 314-bp PCR probe with primers designed towards the 3' end. The probe was used for re-screening the human chondrocyte λ ZAPII cDNA library as described. One 1.6 kb clone (named 92C-2) was isolated, subcloned, and sequenced.

Isolation of genomic clones.

15 Three genomic clones subcloned in the bacteriophage P1 were obtained from Genome Systems, Inc. screening services (St. Louis, MO, USA). One clone containing the gene for CILP was used for isolation of a *XhoI/EcoRI* fragment containing the 3' end. The fragment was subcloned into pBluescript II KS(+) and used for the construction of the expression vectors.

Isolation of cDNA ends.

20 The Marathon cDNA amplification kit (CLONTECH) was used to obtain a library of adaptor-ligated double-stranded cDNA from human articular cartilage. One microgram of poly(A)⁴ RNA was used as a template for the first strand synthesis as described by CLONTECH

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the cells as shown in the two cell systems used for the study of the expression of the protein. Only in the COS-7 cells is a proportion of the precursor protein secreted into the medium, possibly a result from a limited capacity to process the high-level of protein expressed in these cells after transfection.

5 More complete processing was accomplished in the EBNA cells, where the proform of the protein was not secreted into the medium. After processing, a product corresponding to CILP isolated from human articular cartilage (92 kDa) and its smaller variant (82 kDa) were detected by a polyclonal antibody against CILP. The smaller product might be formed by an alternative cleavage site used by the proteases in the expression system. Since this smaller form is not
10 detected in explants of human articular cartilage, it either has a faster turnover or the processing enzymes in human articular chondrocytes are more specific than those in the EBNA cells. Alternative putative cleavage site spanning amino acids 518-519 or 531-535 (Figure 8; SEQ ID NO:2) might be targeted in the cell lines used.

15 CILP is processed by the cells into a single polypeptide chain with a calculated molecular mass of 78,500. It is slightly basic with an isoelectric point of 8.15. It contains 6 putative glycosylation sites, which is consistent with a molecular mass of 92,000, the apparent molecular mass of the protein purified from human articular cartilage after post-translational modifications. The protein contains 30 cysteine residues mostly distributed towards the N-terminal half of the molecule. Due to the special role of cysteine residues for protein structure, the high content of
20 these amino acids indicates a high degree of intrachain disulfide cross-bridging.

Towards the N-terminus of the molecule, there is a stretch of 27 amino acids, from 131 to 157, shown in Figure 8 (SEQ ID NO:2), which is related to the Type I repeats of thrombospondin

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(Lawler and Hynes, 1986, "The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins", *J. Cell Biol.* 103:1635-1648). The N-terminal domain of CILP containing the Type I thrombospondin-like repeat and 16 cysteines is associated with a strikingly hydrophobic region from amino acids 130-225. Following this, a relatively exposed region might lead to another compact, disulfide bonded region between residues 354-445. Another extended region with no cysteines leads to the very basic furin-type cleavage site.

The C-terminal portion of the molecule shows very high similarity to a porcine ectonucleotide pyrophosphohydrolase, NTPPHase (Masuda et al., 1997, *supra*). The human homolog of the nucleotide pyrophosphohydrolase (NTPPHase) that forms the C-terminal of CILP is made of 460 amino acid residues, with a calculated molecular mass of 51,800, and an isoelectric point 8.73. It has 10 cysteine residues and two putative N-glycosylation sites. This is in close agreement with the molecular mass estimate for the catalytically active fragment of the porcine NTPPHase (Masuda et al., 1995, *supra*).

Based on the biochemical characterization of CILP, which has an apparent molecular mass of 92,000, and on the N-terminal sequence reported for a catalytically active proteolytic fragment of the porcine NTPPHase (Masuda et al., 1995, *supra*), it appears that CILP is synthesized as a proform that also contains a protein homologous to a porcine enzyme with NTPPHase activity. In fact the precursor protein has a consensus sequence for precursor cleavage catalyzed by furin (Hosaka et al., 1991, *supra*), *i.e.*, RXK/RR from positions 680 to 683 (RNKR). Directly following the furin consensus cleavage site is the start of the region with homology to the N-terminus of the NTPPHase. The proteolytic fragment of the porcine enzyme

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has a molecular mass of 61,000, which is in agreement with the calculated molecular mass of 51,800 of the translated sequence from base 2302 to base 3684, assuming carbohydrate substitutions at two putative N-glycosylation sites.

The lack of similarities between CILP and other proteins of defined structure did not allow prediction of a secondary structure or any remarkable structural feature or function. The Type I repeat structure is similar to that of the consensus sequence for heparin binding (Guo et al., 1992, "Heparin-binding peptides from the type I repeats of thrombospondin: Structural requirements for heparin binding and promotion of melanoma cell adhesion and chemotaxis", *J. Biol. Chem.* **267**:19349-19355), from residues 131 to 135 (WSPWS; SEQ ID NO:21), but no experimental evidence that CILP actually binds to heparin has yet been obtained.

Example 10: Northern Blotting.

Ten micrograms of total RNA isolated from human articular cartilage were electrophoresed on a 1% formaldehyde-agarose gel, nitrocellulose filter (NitroPure, Micron Separation) and hybridized with random-primed [α -³²P]dCTP probe of 1585-bp *BsmI/EcoRI* fragment from the 92C-1 clone. After hybridization, the membrane was washed twice at 42°C for 15 minutes with 2 x SSC, 0.1% SDS, then with 1 x SSC, 0.1% SDS, and finally with 0.1 x SSC, 0.1% SDS, and exposed to X-ray film (X-OMAT-AR, Kodak).

Northern blotting of total RNA from human articular cartilage, using a 1585 bp *BsmI/EcoRI* fragment from clone 92C-I as the probe, indicated that there is a single mRNA of approximately 4.2 kb (Figure 10). This size agrees well with the total number of nucleotides sequenced, indicating that most of the mRNA is covered by the cDNA clones.

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Example 12: CILP Expression and Detection Using *in vitro* Transcription/translation assays.

The plasmid pBluescript II KS(+) with the expression construct (Figure 9) was transcribed/translated *in vitro* with TNT[®] T3 Polymerase (TNT[®]T3 Coupled Reticulocyte Lysate System, Promega). Supercoiled plasmid (500 ng), was used according to the manufacturer's protocol. The 25 µl reaction mixture contained 12.5 µl of TNT rabbit reticulocyte lysate, TNT reaction buffer, T3 RNA polymerase, RNasin[®] 20 Units, and amino acid mix without methionine, supplemented with [³⁵S]methionine as the radioactive precursor (1000 Ci/mmol). After 90 minutes of incubation at 30°C, labeled polypeptides were separated from free amino acids by ethanol precipitation (10 volumes of ethanol containing 50 mM sodium acetate). Precipitates were resuspended in electrophoresis sample buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 0.002% Bromophenol blue, and 20% Glycerol) with or without 10% 2-mercaptoethanol. The resuspended precipitates were boiled at 100°C for 4 minutes, electrophoresed on 4-16% gradient SDS-polyacrylamide gels according to Laemmli (1970, *supra*), and visualized by fluorography (Chamberlain, 1979, "Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate", *Anal. Biochem.* **98**:132-135). A control reaction with the pBluescript II KS(+) plasmid only was run simultaneously. *In vitro* expression using the cell-free transcription-translation system in the presence of [³⁵S]methionine resulted in detection of a single labeled peptide with an apparent molecular mass of 123,000 (Figure 11A).

Example 13: CILP Expression and Detection *in vivo*.

The processing of the precursor protein was studied by expressing the cDNA construct transiently and stably in two different cell lines.

Transient transfection.

The monkey kidney-derived cell line, COS-7, was grown in F12 Dulbecco's medium, supplemented with 10% fetal calf serum, streptomycin (0.1 mg/ml), and penicillin (100 units/ml) at 37°C in a CO₂ incubator. Semi-confluent cells were transfected using the lipofectamine method, as described by the manufacturer (Life Technologies, Inc.), with either 6 µg of pSVL SV40 vector containing the expression construct or 6 µg of pSVL SV40 vector only. After 72 hours (post transfection) the cells were labeled with [³⁵S]methionine (1000 Ci/mmol) in a methionine-free medium. After 8 hours of labeling, the medium was removed, the cells were washed with PBS, and the cells were lysed with RIPA buffer (0.5% of NP 40, 0.5% Tween 20, 0.5% deoxycholic acid, 0.15 M NaCl, 10 mM KCl, 1 mM EDTA, and 20 mM Tris-HCl, pH7). The medium and the cell extract were independently immunoprecipitated with polyclonal rabbit antiserum raised against CILP. The precipitated products were electrophoresed on a 4-16% gradient SDS-polyacrylamide gel and visualized by fluorography. As a control, the cells were transfected with the respective vector lacking the cDNA insert.

With COS-7 cells, analysis by immunoprecipitation of cell extracts and medium with polyclonal antibodies against CILP showed that the precursor protein was secreted to the medium in three forms (Figure 11B and 11C). One component had an apparent molecular weight slightly larger (*i.e.*, 138 kDa) than the *in vitro* translated product, and probably represents the precursor protein with post-translational modifications. Two smaller components had apparent molecular weights of 92 and 82 kDa, respectively. The 92 kDa component is the same size as the expected size for CILP. In the cell extract the major component was the higher molecular weight product

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with a molecular mass about 60,000 (N2, Figure 12B) was detected. An additional minor component with slightly lower mobility (N1, Figure 12B) was recognized by the antibody. The apparent molecular mass of the major band (60,000; N2) corresponds with that reported for a proteolytic fragment of the porcine NTPPHase (Masuda et al., 1995, *supra*). The presence of these two forms of CILP as well as the putative NTPPHase, differing in size, suggests an alternative cleavage site of the precursor protein. There are basic di- and tri-peptides, between residues 518-519, and 531-535 (Figure 8; SEQ ID NO:2) that might represent cleavage sites for furin-like proteinases. These sites are 164 and 150 amino acids upstream of the furin proteinase cleavage consensus sequence. Cleavage at either of these sites would alter the molecular weight by 16.4 and 15 kDa, respectively. These values correspond well to the difference in apparent molecular weight between the observed CILP forms as well as the NTPPHase forms detected by the respective antibodies, *i.e.*, 92 kDa vs. 82 kDa for CILP. In summary, these experiments show that the precursor protein is fully cleaved by the 293-EBNA cells into two components that are secreted to the medium with no detectable precursor present.

It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. All references cited herein are incorporated in their entirety by reference.